Binding Characteristics of Anti-Rh₀(D) Antibodies to Rh₀(D)-Positive and D⁺ Red Cells

By Nancy A. Cunningham, Arleen P. Zola, Hildegardis L. Hui, Lynne M. Taylor, and Floyd A. Green

The relation of human erythrocyte Rh₀(D) to D⁺ sites is an important unresolved question in the field of immunohematology. To compare the immunological reactivity of Rh(D)-positive and D⁺ erythrocytes, the binding characteristics of two anti-Rh₀(D) antisera to human Rh₀(D)-positive and D⁺ ("low-grade") erythrocytes were studied. ³C-Protein A and direct antibody-labeled techniques were used to generate binding curves and to derive double-reciprocal plots. The results show that the number of antigen sites differ by a factor of 10 to 15 between the Rh₀(D)-positive and D⁺ red cells, but that the dissociation constants between anti-Rh₀(D) and the Rh₀(D) and D⁺ antigens are indistinguishable when studied by the two labeling methods and two different anti-Rh₀(D) antibodies. The extent of binding to 112 different D⁺ samples showed a normal distribution and was independent of apparent phenotype. These data suggest immunologic identity of Rh₀(D) and D⁺ ("low-grade") sites and that the difference between the antigens of Rh₀(D) and D⁺ cells is quantitative only. The data are incompatible with the "missing mosaic" and gene interaction theories of mechanism.

SSTRATTON described a "new" form of the D antigen in 1946, which he called D⁺.¹ Cells possessing this antigen were agglutinated by some but not all anti-D sera used. Later, Race et al² and Renton and Stratton³ studied this antigen further and showed that it was an inherited characteristic. They found that D⁺ red cells were not directly agglutinated by anti-Rh₀(D) serum, but required subsequent antiglobulin addition to show the presence of this antigen.

Several varieties of the D⁺ phenotype have been described. Some examples can be demonstrated by direct agglutination with selected high-protein potentiated incomplete anti-D reagents. These constitute the so-called "high-grade" D⁺s. Other, weaker forms exist that have a strict requirement for two anti-Rh₀(D) antisera to human Rh₀(D)-positive and D⁺ "low-grade" D⁺s.

The relation of human erythrocyte Rh₀(D) to D' sites is an important unresolved question in the field of immunohematology. To compare the immunological reactivity of Rh(D)-positive and D⁺ erythrocytes, the binding characteristics of two anti-Rh₀(D) antisera to human Rh₀(D)-positive and D⁺ ("low-grade") erythrocytes were studied. ³C-Protein A and direct antibody-labeled techniques were used to generate binding curves and to derive double-reciprocal plots. The results show that the number of antigen sites differ by a factor of 10 to 15 between the Rh₀(D)-positive and D⁺ red cells, but that the dissociation constants between anti-Rh₀(D) and the Rh₀(D) and D⁺ antigens are indistinguishable when studied by the two labeling methods and two different anti-Rh₀(D) antibodies. The extent of binding to 112 different D⁺ samples showed a normal distribution and was independent of apparent phenotype. These data suggest immunologic identity of Rh₀(D) and D⁺ ("low-grade") sites and that the difference between the antigens of Rh₀(D) and D⁺ cells is quantitative only. The data are incompatible with the "missing mosaic" and gene interaction theories of mechanism.

MATERIALS AND METHODS

Antiserum. Two high-titered anti-Rh₀(D) sera (C-937 and C-209) were obtained by plasmapheresis from two group AB, Rh₀(D)-negative, hyperimmunized women donors. These sera were tested for IgG subclass with anti-IgG1, -IgG2, -IgG3, and -IgG4 (obtained from Central Laboratory of the Netherlands Red Cross, Amsterdam) using the centrifugation antiglobulin method.¹² Both anti-Rh₀(D) sera were shown to contain all four IgG subclasses. Precipitation of the sera with unlabeled staphylococcal protein A showed that from 25% to 80% of the antibody activity was found in the pellet after centrifugation.

Red Cells. Erythrocytes, Rh₀(D) positive, Rh₀(D) negative, and D⁺, were collected from healthy adults (courtesy of the American Red Cross, Blood Services Buffalo Region) and stored in liquid nitrogen.¹³ The frozen erythrocytes were recovered by the method of Rowe et al.¹⁴ Determination of D⁺ status was based on the fact that these cells were negative with commercial, high-protein modified anti-Rh₀(D) sera and positive with an antiglobulin serum.

Preparation of ³C-Protein A. Radiolabeled protein A was prepared by acetylation with ³C-acetic anhydride as previously described.¹⁵

Preparation of ³C-Labeled Anti-D. Radiolabeled anti-Rh₀(D) was prepared by acetylation with ³C-acetic anhydride as previously described.¹⁵ The magnitude of nonspecific binding was assessed for each preparation and was consistently <3% of the positive.

Preparation of Anti-Rh₀(D)-Sensitized Erythrocytes. Cells were washed three times in 0.02 mol/L, pH 7.4, sodium phosphate buffer in 0.9% sodium chloride (PBS) and were volumetrically made to a nominal 20% suspension in PBS. The accuracy of cell suspensions was verified by means of particle counting in a Coulter Counter, Model F (Coulter Electronics, Hialeah, Fla). The suspensions were then divided into 250-µL portions and incubated for 30 minutes at 37 °C with the appropriate volume of anti-Rh₀(D). For

From the Departments of Medicine and Microbiology, State University of New York at Buffalo, the American Red Cross Blood Services, Buffalo Region, and the Veterans Administration Medical Center, Buffalo.

Supported by Grant No. HL24009 from the National Institutes of Health, the American Red Cross Blood Services, Buffalo Region, and the Veterans Administration Medical Center, Buffalo.

Submitted July 30, 1984; accepted March 25, 1985.

Address reprint requests to Dr Floyd A. Green, State University of New York at Buffalo, 3495 Bailey Avenue, Buffalo, NY 14215.

© 1985 by Grune & Stratton, Inc.

experiments using radiolabeled antibody, 100 μL of a 10% cell suspension were incubated with 14C-labeled anti-Rh0(D) serum. After incubation, the cells were washed three times in PBS.

Papain-treated red cells were prepared using the method of Low15 as modified by Masouredis.16

Measurement of 14C-Protein A Bound to Anti-Rh0(D) Sensitized Erythrocytes. The washed anti-Rh0(D)-sensitized red cells, suspended in 200 μL of 0.1 mol/L, pH 8.6 sodium barbital buffer, were incubated for two hours at room temperature with 1.0 μg of 14C-protein A. After incubation, each sample was washed three times with a total of 10 mL of barbital buffer.

Scintillation Counting. Red cell samples from both the 14C-protein A and 14C-anti-Rh0(D) experiments were dissolved in 1.0 mL of a mixture of equal vol of 95% ethanol and Protosol Tissue and Gel Solubilizer (New England Nuclear, Boston), and decolorized with 0.5 mL of 30% hydrogen peroxide. Fifteen milliliters of Biofluor High Efficiency Emulsifier Cocktail (New England Nuclear) were added to each sample before the addition of 0.5 mL of 0.5N HCl. The radioactivity in all samples was then estimated at 4 °C in a Packard Tri-Carb 460 CD Liquid Scintillation Counter System (Packard Instruments, Division of Amback Industries, Downers, Grove, Ill.).

Data Presentation. The nonspecific binding (to Rh0(D)-negative cells) was subtracted from the dpm bound to Rh0(D)-positive and D* cells. The data are presented as previously reported except that, to avoid making certain assumptions about stoichiometry, the absolute numbers of sites are given only for the 14C-anti-Rh0(D) data. A well-studied Rh0(D)-positive (Rr) control subject (NC.) was used throughout. These cells were known to have a relatively small number of sites for a Rh0(D)-positive (≈4,000 sites per cell) as determined by labeled antibody measurements in our laboratory.

RESULTS

Erythrocytes of the D* phenotype are known to vary widely in their capacity to react with IgG anti-Rh0(D). This variation is shown quantitatively in the results depicted in Fig 1. The magnitude of binding of 14C-protein A ranged from 1% to almost 20% of the dpm bound by an arbitrarily selected standard, a Rr cell. The type of distribution, which approximated a normal probability curve, was found with each of the two anti-Rh0(D) sera used. The binding range was the same for both anti-Rh0(D) antisera. The mean

![Fig 1. The distribution of 14C-protein A bound by D* red cell samples sensitized with two different anti-Rh0(D) sera as a percentage of 14C-protein A bound to anti-Rh0(D)-sensitized Rr red cells. A sample of 47 D* specimens was tested with anti-Rh0(D) (C-937), and an additional 85 were tested with anti-Rh0(D) (C-209).](https://example.com/fig1.png)

![Fig 2. Mean and SE of 14C-protein A bound by anti-Rh0(D) (D*-sensitized D* red cells as a percentage of 14C-protein A bound to anti-Rh0(D)-sensitized Rr red cells. □, Anti-Rh0(D) (D*) serum, C-209; □, Anti-Rh0(D) (D*) serum, C-937.](https://example.com/fig2.png)

protein A binding by serum C-209 was 6.6% (SEM 0.43%) and by C-937, 7.9% (SEM 0.47%).

When rearranged by their statistically probable Rh phenotype as shown in Fig 2, the data of Fig 1 do not indicate a significant association of Rh antigens partnered with the D* bearing haplotype. Although some trends were observed (ie. Rr), in no case was significance reached with either antisera.

A typical binding curve is shown in Fig 3A. The data were further analyzed by means of a double-reciprocal plot as shown in Fig 3B. While the number of antigen sites differ by a factor of approximately 10, the dissociation constants, as obtained from the x-intercepts with serum, C-937, are indistinguishable. The same relationship of binding affinities, as seen in Fig 3A and B, was maintained for the other 22 D* samples (2-Rr, 7-Rr, 13-Rr) and 12 Rh0(D)-positive samples (2-Rfr, 3-Rfr, 2-Rfr, 2-Rfr, 3-Rfr) when binding curves were run with the two anti-Rh0(D) sera in the 14C-protein A system, and analyzed by means of double reciprocal plots. The ratio of the mean dissociation constants of Rh0(D)-positive to D* was 1.13 when 12 Rh0(D)-positive and 22 D* red cell samples were tested with anti-Rh0(D) (C-937) in the 14C-protein A assay system.

To ensure that these binding data did not result from some artifact of the protein A measurement technique, direct measurement of 14C-anti-Rh0(D) (D*) was performed, in experiments analogous to those depicted in Fig 3, A and B. The data are illustrated in Fig 4, A and B. The binding curve and double-reciprocal plot using two different D* cell sources and a single antibody show similar results to those in Fig 3. Analysis gave 3.9 x 10^7 Rh0(D) sites/cell for the Rh0(D)-positive and 3.1 x 10^7 sites for the D* with indistinguishable dissociation constants in the 4 x 10^-4 range for the anti-Rh0(D), C-937.

Binding curves were generated with papain-treated and-untreated Rh0(D)-positive, D*, and Rh0(D)-negative cells and the two anti-Rh0(D) antisera, using 14C-protein A. There was no increase in the amount of 14C-protein A bound to papain-treated cells of any group.

DISCUSSION

According to convention, those D* cells that can only be demonstrated by sensitizing the red cells with incomplete anti-Rh0(D) (D*) follow the addition of antiglobulin sera are...
called low-grade D's, the subject of this present study. Few direct attempts have been made to compare Rh0 (D) and D* cells by labeled antibody methods. Masouredis and Sturgeon found reduced binding of labeled anti-Rh0 (D) to D5 compared with Rh0 (D)-positive cells, but no more binding to D5 than to Rh0 (D)-negative cells. That group also reported an increased binding of anti-Rh0 (D) to papain-digested D* cells; we could not confirm this. Our findings of approximately 310 sites for the D5 samples tested are in agreement with the range of sites (110 to 540) per D* cell reported by Bush et al.7

The data presented here do not support either the missing mosaic or gene interaction explanations for the origin of the low-grade D* phenotype. For the missing mosaic explanation to have been upheld, a variety of dissociation constants would be expected when either of the two anti-Rh0 (D) sera were allowed to react with various cells of the D* phenotype. The gene interaction explanation is not supported because no distinct difference in 14C-protein A binding was found in the association of other Rh antigens (ie, C, c, E, or e) with the D* phenotype. Rather, the data suggest simply a genetically determined lowered density of qualitatively normal receptor sites. Final proof, however, of the identity of Rh0 (D) and D* sites must await purification and characterization of the antigen.

APPENDIX

A brief description of the method of data analysis used in this study may be of value. The specific question relates to the use of double-reciprocal plots to analyze antigen–antibody interaction.

There is total consensus that in essence a mass–law relationship properly describes antigen–antibody interaction, but there have been questions raised as to the most appropriate method of linearization. Double-reciprocal plots were first derived by Lineweaver and Burke to analyze enzyme–substrate interaction. This type of plot has been described by Klotz for analysis of binding in general, and by Day for antigen–antibody interaction, as the Langmuir form of the affinity equation,

\[ \frac{1}{r} = \frac{1}{nK_a} + \frac{1}{r} \]

where \( r \) is the bound antibody, \( c \) is the free antibody, \( n \) is the binding number, and \( K_a \) is the association constant. On plotting \( 1/r \) against \( 1/c \), the ordinate intercept gives the reciprocal of the maximum binding number and the abscissa intercept gives \(-1/K_a\). Day has pointed out that the association constant may in fact be an average of high and low binding antibodies in this and other plots. Specific exami-
nation of the extent of binding constant heterogeneity has been the subject of extensive study, but did not seem pertinent to this present investigation.

Tradition has favored the use of Scatchard plots for immunological data analysis. However, in the past few years, there has been considerable debate in the literature as to the best method to analyze mass-law action. Klotz argued that Scatchard plots can be misleading and recommended a plot of bound vs log-free concentration. Rodbard and Munson argued that double-reciprocal plots are equivalent to Scatchard plots and that none of the available methods of linearization is superior to the other. Lefkowitz and Michel proposed rather that computer-assisted non-linear, least-squares curve-fitting be applied to mass-law action. The analysis of Scatchard plots in the past has been thought to be especially informative when the non-straight line relationships are found. The implication of different shapes of curves has been studied in that connection.

Basu et al. were the first to use double-reciprocal plots to analyze Rh antigen-antibody interactions. Surprisingly, a straight line was obtained. This was also true of our previous studies. Although unexpected in view of known antibody heterogeneity, the results have been uniform for at least four different human antisera. The reason for this linearity is not clear, but was found whether direct 14C anti-Rh0 (D) antibody, or 14C-protein A was used. Even if the present Kv should more properly be referred to as an apparent association constant, the main thrust of this study was to compare internally the binding of the same antibody to two different red cell membrane antigens, Rh(D) and D'. Therefore, this method of data analysis, we feel, is justifiable as well as convenient.

REFERENCES

7. Ceppellini R, Dunn LC, Turri M: An interaction between alleles at the Rh locus in man which weakens the reactivity of the Rh0 factor (D'). Proc Natl Acad Sci USA 15:283, 1955
8. Chown B, Lewis M: Occurrence of D' type of reaction when CDe or cDE is partnered with Cde. Ann Hum Genet 22:58, 1957
16. Masouredis SP: Reaction of 111-In anti-Rh0 (D) with enzyme treated red cells. Transfusion 2:63, 1962
Binding characteristics of anti-Rh0(D) antibodies to Rh0(D)-positive and Du red cells

NA Cunningham, AP Zola, HL Hui, LM Taylor and FA Green